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Alternative long-term markers for the detection of methyltestosterone misuse

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ABSTRACT

Methyltestosterone (MT) is one of the most frequently detected anabolic androgenic steroids in doping control analysis. MT misuse is commonly detected by the identification of its two main metabolites excreted as glucuronide conjugates, 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol. The detection of these metabolites is normally performed by gas chromatography–mass spectrometry, after previous hydrolysis with β-glucuronidase enzymes, extraction and derivatization steps. The aim of the present work was to study the sulphate fraction of MT and to evaluate their potential to improve the detection of the misuse of the drug in sports. MT was administered to healthy volunteers and urine samples were collected up to 30 days after administration. After an extraction with ethyl acetate, urine extracts were analysed by liquid chromatography tandem mass spectrometry using electrospray ionisation in negative mode by monitoring the transition m/z 385 to m/z 97. Three diol sulphate metabolites (S1, S2 and S3) were detected. Potential structures for these metabolites were proposed after solvolysis and mass spectrometric experiments: S1, 17α-methyl-5β-androstan-3α,17β-diol 3α-sulphate; S2, 17β-methyl-5α-androstan-3α,17α-diol 3α-sulphate; and S3, 17β-methyl-5β-androstan-3α,17α-diol 3α-sulphate. Synthesis of reference compounds will be required in order to confirm the structures. The retrospectivity of these sulphate metabolites in the detection of MT misuse was compared with the obtained with previously described metabolites. Metabolite S2 was detected up to 21 days after MT administration, improving between 2 and 3 times the retrospectivity of the detection compared to the last long-term metabolite of MT previously described, 17α-hydroxy-17β-methylandrostan-4,6-dien-3-one.

1. Introduction

Methyltestosterone (MT, 17β-hydroxy-17α-methylandrost-4-en-3-one) is a synthetic anabolic androgenic steroid (AAS) used in sports to increase muscular mass and to improve performance. MT is one of the most frequently detected AAS in doping analysis [1]. Therefore, antidoping control laboratories need to develop strategies to improve the detection of its misuse.

The use of MT is normally screened by the detection of the two main metabolites, 17α-methyl-5α-androstan-3α,17β-diol (M1) and 17α-methyl-5β-androstan-3α,17β-diol (M2) (Fig. 1) which were firstly described by Rongone and Segaloff [2]. The detection of these metabolites is commonly performed by gas chromatography–mass spectrometry (GC–MS), with previous hydrolysis with β-glucuronidase and derivatization steps [3–5]. Hydrolysis is compulsory since M1 and M2 are mainly excreted as conjugates with glucuronic acid while the derivatization and subsequent GC–MS analysis is required since M1 and M2 are poorly detectable by other techniques like liquid chromatography-tandem mass spectrometry (LC–MS/MS) due to the limitation of ionizable groups [6].

In the doping control field, the best marker for the detection of a drug administration is not always the most abundant metabolite but the one which can be detected for the longest period (the so-called long-term metabolites). Therefore, metabolic studies are helpful in order to detect alternative long-term metabolites. During the last years, the use of LC–MS/MS has allowed the detection of several long-term metabolites for some AAS like stanozolol, methandienone or testosterone [7–9]. In the case of MT, other minor metabolites detectable by LC–MS/MS like 17α-hydroxy-17β-methylandrost-4,6-dien-3-one (M3) or 17α-hydroxy-17β-methylandrost-4-en-3-one (M4) [10] have been reported, (Fig. 1). In addition to that, other metabolites like 17β-hydroxymethyl-17α-methyl-18-norandrosta-4,13-dien-3-one (M5) [11] or 17β-methyl-5α-androstan-3α,17α-diol (M6) and 17β-methyl-5β-androstan-3α,17α-diol (M7) [12] have been reported by using GC–MS/MS or GC–MS after β-glucuronidase hydrolysis (Fig. 1). While M5 is excreted as glucuronide, the occurrence of M3, M4, M6 and M7 in urine can be explained by epimerization at the C17 after conjugation with a sulphate moiety. 17β-Sulphates are spontaneously hydrolysed in urine to several dehydratation products, and to the 17α-hydroxy-17β-methyl epimers [12–14] (Fig. 1). Among all those metabolites, it was demon-

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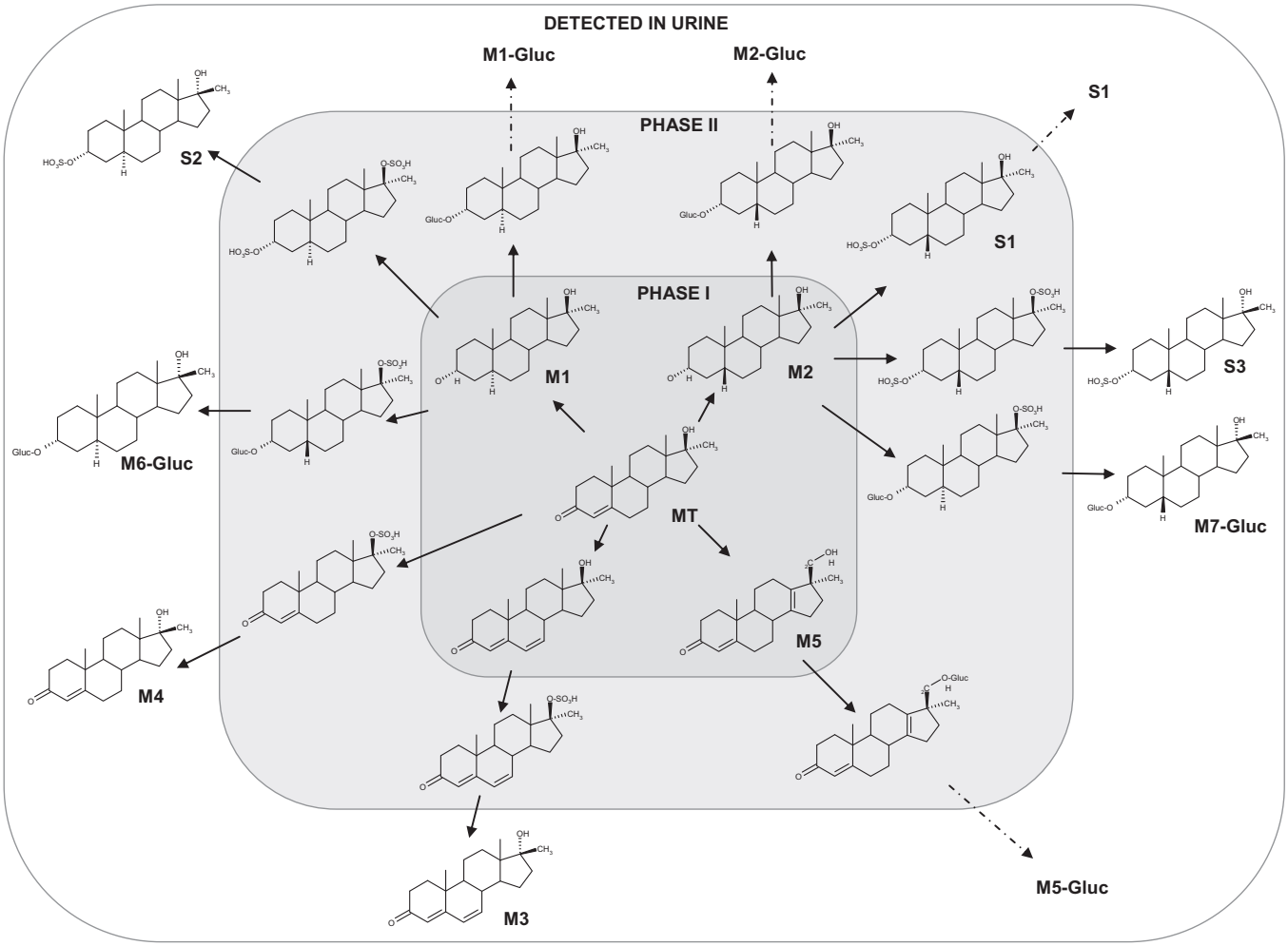

Fig. 1. Location of the reported metabolites S1, S2 and S3 in the metabolic pathways for methyltestosterone based on previously reported results [2,3,10–12].

strated that the LC–MS/MS detection of M3 provides the highest retrospectivity [10]. This fact shows the potential usefulness of metabolites conjugated with sulphates for the long-term detection of MT misuse. Sulphate metabolites are known to be important for some endogenous steroids and they have also been described for exogenous AAS [4,15–17]. However, a comprehensive study of this phase II biotransformation for MT has not been performed.

The use of LC–MS/MS is gradually becoming more important for the detection of phase I and II metabolites of doping agents [7–10,17–23]. Recent studies by our group showed the potential of LC–MS/MS for the direct detection of sulphate metabolites of other AAS. Minor metabolites of boldenone excreted as sulphate conjugates (0.01% of the administered dose) did provide the same retrospectivity than the main glucuronide metabolites [18].

The aim of this work was to study metabolites of MT conjugated with sulphate using LC–MS/MS analysis, and to evaluate their potential to improve the detection of MT compared with previously described metabolites.

2. Experimental

2.1. Chemicals and reagents

17α-Methyl-5α-androstan-3α,17β-diol (M1), 17α-methyl-5β-androstan-3α,17β-diol (M2) and etiocholanolone sulphate were obtained from NMI (Pymble, Australia). 17α-Methyl-5α-androstan-3β,17β-diol and 17α-methyl-5β-androstan-3β,17β-diol were purchased from Research Plus, Inc. (Bayonne, NJ, USA). Boldenone was obtained from Sigma (Steinheim, Germany).

Tert-butyl methyl ether (TBME, HPLC grade), ethyl acetate (HPLC grade), acetonitrile and methanol (LC gradient grade), formic acid (LC/MS grade), potassium carbonate, sulphuric acid, sodium hydroxide, di-sodium hydrogen phosphate, sodium hydrogen phosphate, sodium chloride, ammonia hydroxide, ammonium chloride, ammonium iodide, and 2-mercaptoethanol (all analytical grade) were purchased from Merck (Darmstadt, Germany). Ammonium formate (HPLC grade) and the alkane standard mixture (C20–C40) were obtained from Sigma–Aldrich (Steinheim, Germany). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Macherey-Nagel (Düren. Germany). β-Glucuronidase from *Escherichia coli* K12 was obtained from Roche Diagnostics (Mannheim, Germany). Detectabuse™ XAD-2 extraction columns were purchased from Biochemical Diagnostics, Inc. (Edgewood, NY, USA). Milli Q water was obtained by a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

2.2. Sample preparation

2.2.1. GC–MS analysis of M1 and M2

One hundred nanograms per millilitre of boldenone (internal standard, ISTD) were added to urine samples (5 mL) which were passed through XAD-2 columns previously conditioned with 2 mL methanol and 2 mL water. The column was washed with

2 mL water and the analytes were eluted with 2 mL methanol. The methanolic extract was evaporated to dryness under a stream of nitrogen in a water bath at 50 °C and reconstituted with 1 mL of sodium phosphate buffer (0.2 M, pH 7). Enzymatic hydrolysis was performed by adding 30 μL of β-glucuronidase from *E. coli* and incubating the mixture at 55 °C for 1 h. After the sample reached room temperature 250 μL of 5% K_2CO_3 solution were added and the mixture was extracted with 6 mL of TBME by shaking at 40 movements per minute (mpm) for 20 min. After centrifugation (3000*g*, 5 min), the organic layer was separated and evaporated to dryness under a stream of nitrogen in a water bath at 40 °C. The dry extract was derivatized by adding 50 μL of a mixture of MSTFA/NH_4I/2-mercaptoethanol (1000:2:6, v/w/v) mixture and incubating at 60 °C for 20 min. After incubation, the derivatized extracts were transferred to injection vials and 2 μL were analysed by GC–MS.

2.2.2. LC–MS/MS analysis of M3

M3 was analysed in the free fraction, urine samples (5 mL) were spiked with boldenone (ISTD) to a final concentration of 100 ng mL^{-1}. Afterwards, 250 μL of 25% K_2CO_3 solution were added, and a liquid–liquid extraction was performed with 6 mL of TBME and shaking at 40 mpm for 20 min. After centrifugation (3000*g*, 5 min), the organic layer was separated and evaporated to dryness under a stream of nitrogen in a water bath at 40 °C. The extract was reconstituted with 100 μL of a mixture of water:acetonitrile (1:1, v/v) and 10 μL were analysed by LC–MS/MS.

2.2.3. Direct LC–MS/MS analysis of diol sulphate metabolites

Sample preparation was based on a previously described procedure [24,25]. Briefly, urine samples (5 mL) were spiked with boldenone (ISTD) to a final concentration of 100 ng mL^{-1}, and the pH adjusted to pH 9.5 by addition of 100 μL of a buffer (5.3 M ammonium chloride/ammonia). Then, sodium chloride (1 g) was added to promote the salting-out effect and the samples were extracted with 8 mL of ethyl acetate by shaking at 40 mpm for 20 min. After centrifugation (3000*g*, 5 min), the organic layer was evaporated to dryness under a nitrogen stream in a water bath at 40 °C. The extracts were reconstituted with 100 μL of a mixture water:acetonitrile (9:1, v/v) and 10 μL were analysed by LC–MS/MS.

2.2.4. Fraction collection and solvolysis

For metabolite characterisation, a LC fractionation of urine samples was performed. Sample preparation described above in Section 2.2.3. was applied to 20 replicates of 5 mL of urine. Prior to evaporation, the organic phases of all the replicates were combined, evaporated, reconstituted and injected in a single injection. Fractions corresponding to the expected retention time (RT ± 0.2 min) of the peaks of each metabolite were manually collected in pre- and post-administration samples. LC fractions were evaporated, and a solvolysis was performed using a procedure described elsewhere [17,26]. Briefly, fractions were reconstituted with 4 mL of ethyl acetate/methanol/sulphuric acid (80:20:0.06, v/v/v) and incubated at 55 °C for 2 h. Extracts were neutralized with 60 μL of 1 M NaOH and evaporated to dryness. The residues were reconstituted in 1 mL of sodium phosphate buffer (0.2 M, pH 7) and 250 μL of 5% K_2CO_3 solution were added. The extraction was performed with 6 mL of TBME by shacking at 40 mpm for 20 min. After centrifugation (3000*g*, 5 min), the organic layers were separated and evaporated. The dry extracts were derivatized by adding 50 μL of a mixture of MSTFA/NH_4I/2-mercaptoethanol (1000:2:6, v/w/v) and incubating at 60 °C for 20 min. After incubation, the derivatized extracts were transferred to injection vials and 1 μL was analysed by GC–MS.

2.3. LC–MS/MS instrumental conditions

Chromatographic separations were carried out on a Waters Acquity UPLC™ system (Waters Corporation, Milford, MA, USA) using an Acquity BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm particle size). The column temperature was set to 55 °C. Separation was performed at a flow rate of 0.3 mL min^{-1}.

The LC instrument was coupled to a Quattro Premier XE triple quadrupole mass spectrometer (Micromass, Waters Corporation, Milford, MA) with an electrospray (Z-spray) ionisation source. Source conditions were fixed as follows: lens voltage, 0.2 V; source temperature 120 °C; desolvation temperature, 450 °C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 1200 L/h. In positive mode the capillary voltage was set at 3 kV, whereas in negative mode the capillary voltage was set at 2.5 kV. Nitrogen was used as desolvation gas and argon was used as collision gas.

2.3.1. Analysis of 17-methylandrostandiol sulphate metabolites of MT

The mobile phase consisted of deionized water with 0.01% formic acid (solvent A) and acetonitrile with 0.01% formic acid (solvent B) using a gradient pattern: from 0 to 0.5 min, 5% B; from 0.5 to 6 min, to 50% B; from 6 to 7 min, to 90% B, during 0.2 min, 90% B; from 7.2 to 7.4 min, to 5% B; from 7.4 to 10 min, 5% B. Data acquisition was performed in selected reaction monitoring (SRM) mode (Method 1, Table 1).

2.3.2. Fraction collection

Each metabolite was isolated by LC fractionation. The mobile phase consisted of water with 0.01% formic acid (solvent A) and acetonitrile with 0.01% formic acid (solvent B). The following gradient was applied: from 0 to 0.5 min, 15% B; from 0.5 to 9 min, to 50% B; from 9 to 12 min, to 80% B, during 0.3 min, 80% B; from 12.3 to 12.5 min, to 5% B; from 12.5 to 15 min, 15% B. The eluent of the column was divided into two (approximate split 1:1), one part was collected and the other was sent to the MS in order to confirm that the fraction contained the selected metabolite. Data acquisition was performed in SRM mode (Method 1, Table 1).

2.3.3. Analysis of metabolite M3

The mobile phase consisted of water with 1 mM ammonium formate and 0.01% formic acid (solvent A) and methanol with 1 mM ammonium formate and 0.01% formic acid (solvent B) using a gradient pattern: from 0 to 1.5 min, 50% B; from 1.5 to 7 min, to 95% B; during 1 min, 95% B; from 8 to 8.5 min, to 50% B; from 8.5 to 12 min, 50% B. Data acquisition was performed in SRM mode (Method 2, Table 1).

Table 1
Mass spectrometric methods for the detection of methyltestosterone metabolites.

Method	Analytes	Instrument	Ionisation mode	Ion[a] (m/z)	Product ion (m/z)	CV (V)	CE (eV)
1	Boldenone (ISTD)	LC–MS/MS	ESI+	287	121	25	20
	S1, S2, S3		ESI−	385	97	60	40
2	Boldenone (ISTD)	LC–MS/MS	ESI+	287	121	25	20
	M3		ESI+	301	183	25	35
				301	210	25	35
				301	225	25	30
3	Boldenone (ISTD)	GC–MS	EI	430			
				415			
				206			
	M1, M2		EI	435			
				345			
				143			

[a] Precursor ion for LC–MS/MS methods and measured ions for GC–MS methods.

2.4. GC–MS instrumental conditions

GC–MS analyses were carried out using a 7890N gas chromatograph coupled to a 5975 MSD (Agilent Technologies, Palo Alto, CA, USA). The steroids were separated on an HP-Ultra1 cross-linked methyl silicone column, 16.5 m × 0.2 mm inner diameter, film thickness 0.11 μm (J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a constant pressure of 23 psi.

2.4.1. Identification of 17-methylandrostandiol metabolites

The gas chromatograph temperature was ramped as follows: initial 70 °C for 0.7 min, then 50 °C/min to 180 °C, then 0.5 °C/min to 192 °C, then 40 °C/min to 310 °C for 3 min. The total run time was 32.85 min. A 1 μL aliquot of the final derivatized extract was injected into the system operated in split mode (split ratio 1:20). The injector and transfer lines were kept at 280 °C. Data acquisition was performed in scan mode by scanning the mass range from m/z 50 to m/z 600.

For the calculation of Kovats indices, 8 μL of the alkane standard mixture (C20–C40) were added to the 50 μL derivatized extracts, after mixing.

2.4.2. Analysis of metabolites M1 and M2

In order to evaluate the retrospectivity achieved, the conventional screening method for the detection of M1 and M2 was also assayed. In this case, the gas chromatograph temperature was ramped as follows: initially 180 °C, increased to 230 °C at 3 °C/min, and thereafter increased to 310 °C at 40 °C/min and kept at 310 °C for 3 min. The injector and transfer lines were kept at 280 °C. A 2 μL aliquot of the final derivatized extract was injected into the system operated in split mode (approximate split ratio 1:20). Data acquisition was performed in selected ion monitoring mode (SIM) (Method 3, Table 1).

2.5. Excretion studies

Urine samples obtained from two excretion studies involving the administration of a single dose of 10 mg of methyltestosterone to healthy volunteers were analysed (subject A, male, caucasian, 54 years, 90 kg; subject B, male, caucasian, 25 years, 65 kg). The clinical protocol was approved by the Local Ethical Committee (CEIC-IMAS, Institut Municipal d'Assistència Sanitària, Barcelona, Spain). The urine samples were collected before administration and daily up to 13 days after administration in the first study and up to 30 days after administration in the second study. Urine samples were stored at −20 °C until analysis.

3. Results

3.1. Detection of putative 17-methylandrostandiol metabolites of MT conjugated with sulphate

In order to detect potential 17-methylandrostandiol-mono-sulphate metabolites of MT, a SRM method using the transition from m/z 385 to m/z 97 in negative ionisation mode was developed (Method 1, Table 1). The method was applied to samples collected before and after administration of MT.

Three main potential sulphate metabolites (RT: 5.65, 7.16 and 7.27 min) were detected in post-administration samples. No peaks at the same RT were observed in pre-administration samples (Fig. 2). The potential metabolites were labelled as S1, S2 and S3 and the studies were conducted in order to elucidate their structure. Additionally, other minor peaks at RT of 6.73, 6.82, 7.36 and 7.52 min were observed. These peaks were not comprehensively studied due to their low abundance.

3.2. Identification of sulphate metabolites

In order to identify the structure of the sulphate metabolites, LC–MS analyses in full scan, both in positive and negative ionisation modes, were carried out. In positive mode, no signal was observed for any of the metabolites. In negative mode, only one peak was observed at the expected RTs in all cases, an ion at m/z 385 was found, corresponding to the $[M-H]^-$ ion. Product ion scan spectra of m/z 385 at different collision energies exhibited only one abundant ion at m/z 97, for the three metabolites.

Isolation of each metabolite by LC was performed at the following RTs: 6.75 min for metabolite S1, 9.57 min for metabolite S2 and 9.81 min for metabolite S3. The solvolysis procedure was applied to each fraction in order to release the corresponding phase I metabolite. Afterwards, silylation was conducted, and the extracts were analysed by GC–MS in scan mode. RTs and Kovats indices of the peaks obtained are listed in Table 2. Electron ionisation mass spectra are shown in Fig. 3.

Electron ionisation mass spectra of the bis-O-TMS derivatives of the 17α-methylandrostandiols commercially available are shown

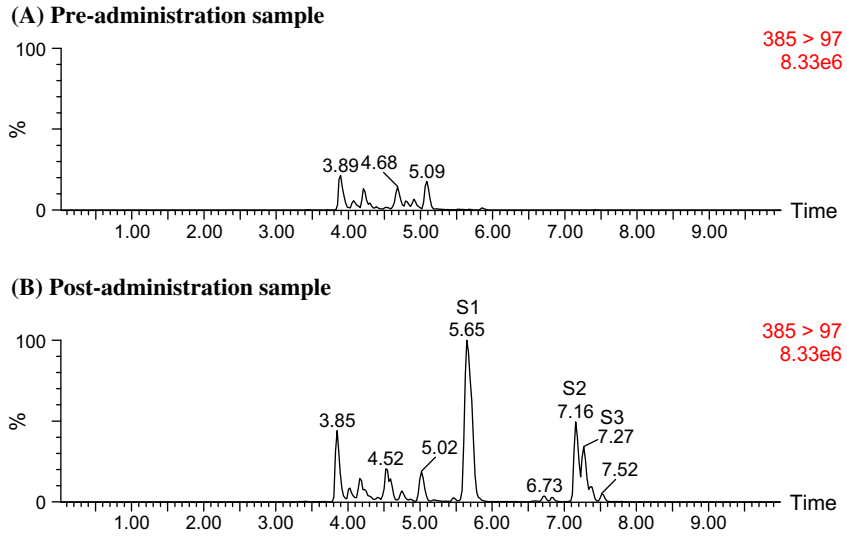

Fig. 2. SRM chromatograms (transition 385 > 97) for pre-administration sample (A) and sample collected after administration of methyltestosterone (B).

Table 2
Retention times (RT) and calculated Kovats indices for the bis-O-TMS derivatives of steroids released after solvolysis of S1, S2 and S3 and the bis-O-TMS derivatives of MT metabolites commercially available, and Kovats indices for the bis-O-TMS derivatives of all possible MT metabolites, reported in literature [12].

Name	RT (min)	Calculated Kovats indices	Reported Kovats indices [12]
17α-Methyl-5β-androstan-3β,17β-diol	24.65	2580	2629
17α-Methyl-5α-androstan-3α,17β-diol (M1)	24.97	2584	2634
17α-Methyl-5β-androstan-3α,17β-diol (M2)	25.66	2594	2637
17α-Methyl-5α-androstan-3β,17β-diol	28.18	2692	2739
17β-Methyl-5β-androstan-3β,17α-diol	–	–	2479
17β-Methyl-5α-androstan-3α,17α-diol	–	–	2482
17β-Methyl-5β-androstan-3α,17α-diol	–	–	2466
17β-Methyl-5α-androstan-3β,17α-diol	–	–	2626
Phase I metabolite released from S3	16.48	2425	–
Phase I metabolite released from S2	17.27	2441	–
Phase I metabolite released from S1	25.66	2594	–

in Fig. 4. All of them have 17β-hydroxy-17α-methyl structure: M1, M2 and their 3β-hydroxy analogues, 17α-methyl-5α-androstan-3β,17β-diol and 17α-methyl-5β-androstan-3β,17β-diol. RTs and Kovats indices obtained for these standards, which are in agreement with previously reported values [12] are listed in Table 2.

3.3. Excretion study samples

In order to detect the most significant MT metabolites (M1, M2 and M3) currently monitored, extracts from urine samples obtained before and after administration of a single oral dose of MT were analysed by the described methods.

The GC–MS analysis of the main metabolites, M1 and M2 excreted as glucuronoconjugates, showed a detection window up to 6 days after the administration of the drug in both volunteers. Particularly, M1 was detected up to 4 days in both administration studies, and M2 was detected up to 5 days in the first study and up to 6 days in the second study (Fig. 5).

LC–MS/MS analysis showed that detection of M3 was possible 8 days after the drug intake in the first study and up to 9 days in the second one (Fig. 5).

Regarding the sulphate metabolites, S1 was detected up to 6 days in the first study and up to 7 days in the second study. S2 was detected in all post-administration samples in the first study (last sample collected at day 13th) and up to 21 days in the second study. S3 was detected up to 6 days in the first study and up to 8 days in the second study (Fig. 5).

The maximum concentrations of all the metabolites were obtained in samples collected between 24 and 36 h after administration. For metabolites M1 and M2, the maximum concentrations were in the range from 250 to 400 ng mL^{-1}. Due to the lack of analytical standards, concentrations of S1, S2 and S3 were estimated using a calibration curve of etiocholanolone sulphate and they were in the range from 30 to 230 ng mL^{-1}.

In Fig. 6, chromatograms of the characteristic transitions of metabolites M3, S1, S2 and S3 corresponding to the analysis of a pre-administration sample and samples collected on days 5, 8, 12 and 20 are presented.

4. Discussion

Phase II metabolic reactions of AAS have been traditionally studied by using specific hydrolysis to unconjugated metabolites that were subsequently determined by GC–MS or LC–MS/MS [3,4,7,8]. Most AAS metabolic studies used enzymes with β-glucuronidase activity, thus mainly conjugates with glucuronic acid have been systematically studied. Due to the difficulties in the hydrolysis of sulphates, this type of conjugation has not been comprehensively studied [17,27–29]. In spite of these limitations, it is well known that sulphate metabolites are important for some naturally occurring steroids [15,16] and for some exogenous AAS [4,17,29,30]. The importance of sulphate conjugates on MT metabolism has also been demonstrated by the identification of different metabolites with a 17α-hydroxy-17β-methyl structure. These metabolites result from the epimerization at C17 as a consequence of the conjugation with a sulphate of the hydroxyl group at C17 [12–14]. Some of these metabolites are excreted for longer times than the most abundant metabolites excreted as glucuronoconjugates, indicating the interest of sulphate conjugates to improve the retrospectivity for the detection of MT misuse.

In the present study, the direct detection of 17-methylandrostandiol sulphate metabolites of MT was evaluated by LC–MS/MS using the transition m/z 385 to m/z 97. The precursor ion of this transition corresponds to the $[M-H]^-$ of MT metabolites completely reduced. The product ion at m/z 97 corresponds to the anion HSO_4^- which is a common product ion to several sulphate conjugates [18,31]. By using this specific and sensitive SRM method, seven potential 17-methylandrostandiol sulphates were detected. However, relatively large concentrations are required for the characterisation of these compounds. Therefore, the identification was only focused on the most abundant peaks (S1, S2 and S3).

No structural information could be obtained using product ion mass spectra of $[M-H]^-$ ion of S1, S2 and S3, since the only originated fragment was m/z 97. Structural information of sulphate conjugates of steroids can be obtained using product ion scan in positive ion mode, where after the loss of the sulphate moiety different ions related with the steroid structure can be observed [18]. Nevertheless, for S1, S2 and S3 no peaks were detected in positive ion mode, due to the lack of ionizable groups. Therefore, the identification of the metabolites had to be based on the isolation of each metabolite and the subsequent analysis by GC–MS, after solvolysis and enol-TMS derivatization.

4.1. Identification of 17-methylandrostandiol sulphates

4.1.1. Metabolite S1

In GC–MS analysis, a peak at the same RT of M2 was obtained for S1 after solvolysis of the LC fraction and enol-TMS derivatization (Table 2). Electron ionisation mass spectrum (Fig. 3A) was identical to the obtained for M2 (Fig. 4). Similar electron ionisation mass spectra were obtained for the bis-O-TMS derivatives of the four metabolites commercially available (Fig. 4). The most abundant ion at m/z 143 corresponds to the cleavage of D-ring [3]. Other minor ions at m/z 255 and 345 corresponding to losses of O-TMS moieties and m/z 435 corresponding to the loss of a methyl group were also observed. The only significant difference among them was the ion at m/z 270 which was more abundant in 5β diol metabolites than in the 5α ones.

Additionally, the Kovats index calculated for S1 after solvolysis and derivatization was identical to the obtained for M2 in the same conditions (Table 2). These facts confirmed that S1 is 17α-methyl-5β-androstan-3α,17β-diol conjugated with a sulphate. 17β-Sulphate of the tertiary hydroxyl group is sterically influenced and decomposes in urine to yield several dehydration products, among

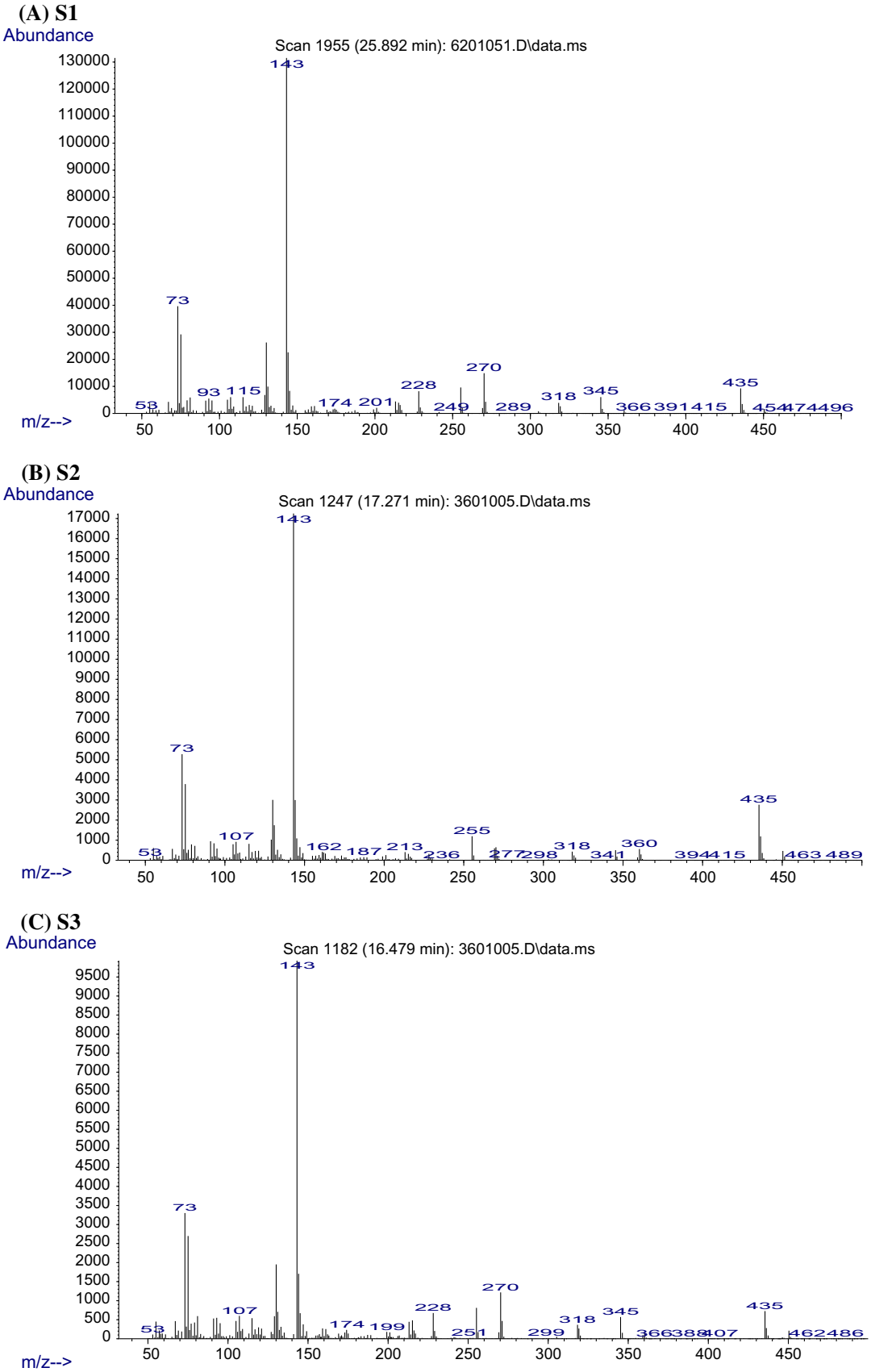

Fig. 3. Electron ionisation mass spectra of the bis-O-TMS derivatives of the phase I metabolites released after solvolysis of S1 (A), S2 (B) and S3 (C).

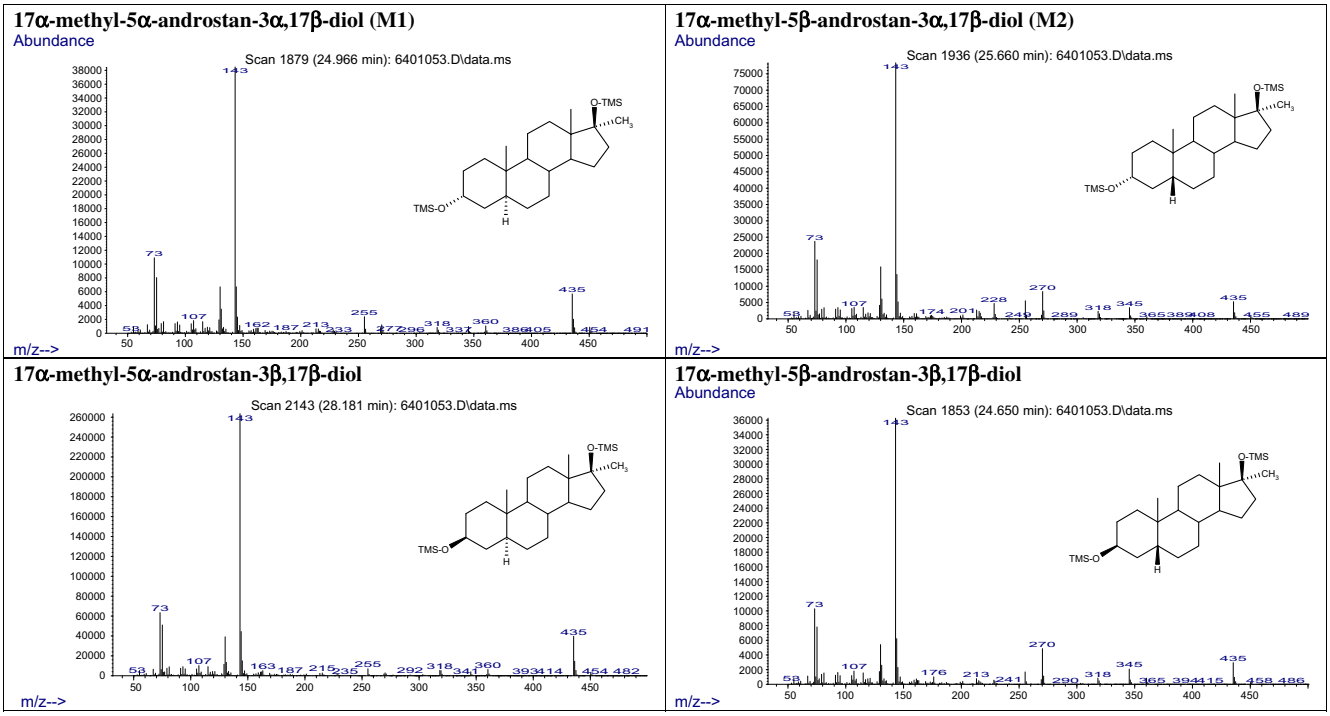

Fig. 4. Electron ionisation mass spectra of the bis-O-TMS derivatives of the reduced MT metabolites, commercially available as standards.

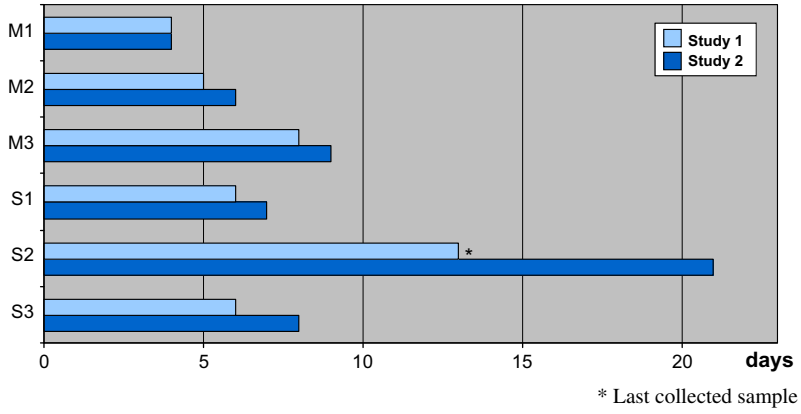

Fig. 5. Detection times of the different metabolites of MT obtained in the two excretion studies.

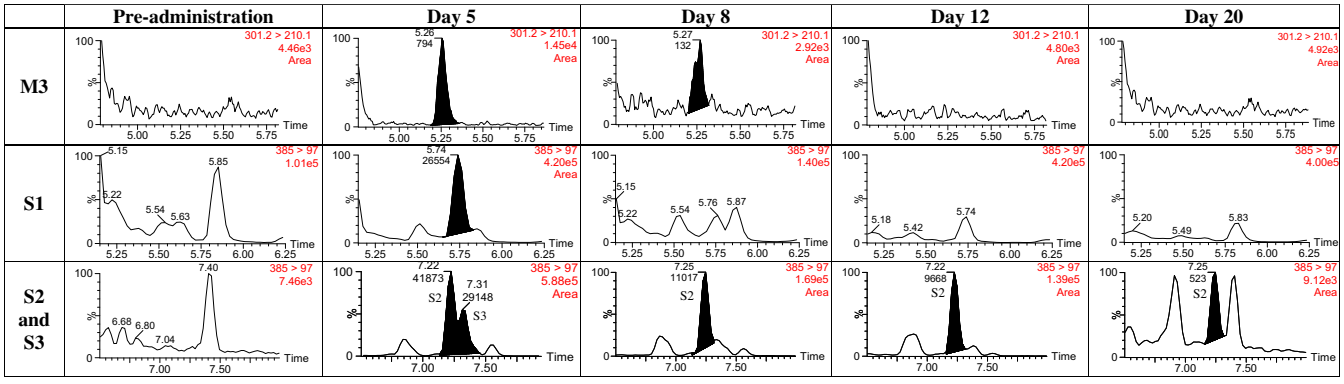

Fig. 6. SRM chromatograms (transitions corresponding to metabolites M3, S1, S2 and S3) of a pre-administration sample and samples collected at 5, 8, 12 and 20 days post-administration.

them, the corresponding 17-epimeric isomer (17α-hydroxy-17β-methyl) [4,12–14]. Therefore, since S1 has been shown to be stable in urine, the position of the sulphate group in S1 is postulated at C3. Synthesis and comparison with reference material would be needed for the definitive confirmation of the structure.

4.1.2. Metabolite S2

Electron ionisation mass spectrum of the bis-O-TMS derivative of the steroid released after solvolysis of S2 (Fig. 3B) was similar to that of the bis-O-TMS derivative of M1 (Fig. 4). But the RT (17.27 min) was clearly different from that of M1 (Table 2). In the GC–MS analysis, the unconjugated steroid obtained from S2 eluted 7 min earlier than any 17α-methylandrostandiol, whereas by LC–MS/MS S2 eluted 2.8 min later than S1 (17α-methyl-5β-androstan-3α,17β-diol 3α-sulphate). As described by Schänzer et al. [12], the elution order when using a reverse phase LC column of the epimerization products is inverse to the elution order of their corresponding TMS derivates in a methylpolisiloxane GC separation. Based on these results, we propose 17β-methyl-5α-androstan-3α,17α-diol sulphate as the potential structure for S2.

As stated previously, 17β-sulphate of the tertiary hydroxyl group is not stable in urine as they are prone to suffer a 17-epimerization. Therefore we postulate that S2 is the result of the 17-epimerization of a disulphate (Fig. 1). The spontaneous release of the sulphate at C17 promotes its epimerization and the remaining sulphate group for S2 is at C3. Synthesis of reference compounds will be required in order to confirm the structures.

4.1.3. Metabolite S3

Similarly to S2, the bis-O-TMS derivative of the steroid released after S3 solvolysis elutes 8 before than any of the four 17α-methylandrostandiol assayed in the GC–MS analysis, whereas by LC–MS/MS S3 eluted 3 min later than S1, suggesting an epimerization at C17 position. Electron ionisation mass spectrum (Fig. 3C) was similar to that of M2, with a prominent ion at m/z 270, characteristic of 5β-metabolites (Fig. 4). Based on these results, we propose 17β-methyl-5β-androstan-3α,17α-diol sulphate as the most feasible structure for S3. Analogously to S2, S3 is the result of the 17-epimerization of a disulphate (Fig. 1), hence the position of the remaining sulphate group is postulated at C3. Synthesis of reference compounds will be required in order to confirm the structures.

4.2. Retrospectivity of sulphate metabolites to detect MT misuse

M1, M2 and M3 are the metabolites currently used as markers of MT administration. M1 and M2 are the most abundant metabolites, M3 having the longest retrospectivity. Detection times of M1, M2, and M3 obtained in the two excretion studies are in agreement with previously reported data using similar analytical approaches [10].

For the sulphates S1 and S3, the detection times were similar to those obtained with M2 and M3, respectively. However, S2 substantially improved the retrospectivity for the detection of MT misuse (between 2 and 3 times when compared to the last long-term metabolite reported, M3) (Figs. 5 and 6).

Our results indicate a similar phase II metabolic pattern for MT compared to other AAS [18,29,30]. Glucuronoconjugation is the most important phase II metabolic reaction, and glucuronoconjugates are excreted rapidly after administration. Once the excretion of glucuronides is declining, the sulphates became predominant and, although from a quantitative point of view they are excreted in lower amount than glucuronides, their longer excretion times make them of great interest to increase the retrospectivity of the detection of the misuse of some AAS.

In summary, three novel sulphate metabolites of MT have been directly detected by LC–MS/MS. The structures of these metabolites have been proposed. Two of them, metabolites S2 (17β-methyl-5α-androstan-3α,17α-diol 3α-sulphate) and S3 (17β-methyl-5β-androstan-3α,17α-diol 3α-sulphate) are result of the initial formation of a disulphate. Therefore, the results demonstrate the importance of sulphatation as a phase II metabolic pathway for MT and, probably, for other AAS, as indirectly known by the existence of metabolites resulting from C17 epimerization.

The interest of sulphate conjugates as long-term metabolites of MT has also been proved. Based on our results, one of the sulphated metabolites, S2 (17β-methyl-5α-androstan-3α,17α-diol 3α-sulphate) can significantly improve the retrospectivity of the detection of MT misuse and the inclusion of its measurement in screening methods will be useful for doping control purposes. Since interindividual variability is expected in the metabolism of AAS, the occurrence of S2 in every human urine sample after MT administration should be confirmed. For this purpose, it would be advisable to perform additional administration experiments using subjects differing in factors like ethnic group, sex or age.

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